Abnormal Muscle and Hematopoietic Gene Expression May be Important for Clinical Morbidity in Primary Hyperparathyroidism

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Abstract

Primary hyperparathyroidism (PHTP) is characterized by overproduction of parathyroid hormone and an increase in the level of serum ionized calcium. The most common cause of excess hormone production is the development of a benign tumor (adenoma) in one of the parathyroid glands.Adenoma accounts for 80-90% percent of all patients with PHTP. Muscular fatigue, constipation ,weakness, lethargy, neuropsychiatric, neuromuscular and cardiovascular manifestations, renal stones, osteoporosis, ostopenia, peptic ulcers, pancreatitis and gallstones are some of the symptoms of PHTP. Nowadays many patients with PHTP are discovered in their asymptomatic phase, especially in Western Europe and North Amerika(2) probably by routine screening This project was aimed to characterize changes in muscle and hematopoietic gene expression in patients with reversible mild PHPT during illness and after parathyroidectomy and possibly link molecular pathology to the symptoms. The transcriptional activity was analysed in biopsies obtained before and one year after parathyroidectomy in 7 patients with mild PHTP comparing bone marrow and muscle genes. My task was to quantify the distribution of PTH receptors (PTHR1 and PTHR2) using real time RT-PCR in unrelated persons to define PTH target tissues and quantify PTH receptors in prathyroidectomized patients. These studies showed that PTHR1 and PTHR2 were more abundantly expressed in muscle and brain than in hematopoietic cells. It is possible that sustained stimulation of PTH receptors in brain,muscle, heart and hematopoietic cells because of elevated serum levels of PTH lead to alterations in gene expression and gives rise to symptoms like muscle fatigue, weakness, cardiovascular pathology and neuropsychiatric manifestations.

Introduction

PHPT is one of the most common endocrine disorders and is usually caused by benign tumor in one of the parathyroid gland(parathyroid adenoma). Hyperplasia is present 10%-20% of cases of primary hyperparathyroidism. This enlargement can occur of all

four parathyroid glands and in some cases enlargement may be grossly apparent in only one or two gland. More rare is conditions with two parathyroid adenomas while having two normal gland. PHPT is more common in adults then in young people and more common in women than in men.

Many reports (2,14,23,24,42,43) emphasize the change in the clinical presenting picture form renal, bone related and gastrointestinal manifestations to psychological and psychiatric disorders as feelings of muscular weakness, apathy, cardiovascular manifestations and ill defined mental symptoms. An increased cardiovascular morbidity has been reported even in mild disease(2), leading to over-presentation of cardiac death in patients with symptomatic PHTP both before and after parathyroidectomy suggesting development of non-reversible changes.

A study shows that adolescents and young adults have a clinical profile that differs from older patients(29). PHPT is rare condition in this age group. The report showed a non-specific symptomatology in adolescent and young adult patients with primary hyperparathyroidism. Fatigue, exhaustion, weakness and lethargy were the most common symptoms. Other symptoms were constipation, nephrolithiasis, polydipsia?, bone pain, palpitations, joint pain, polyuria or nocturia, pruritis, hypertension, depression and many other symptoms. It seems that a variety of symptoms and other organ manifestations are associated with PHTP.

Nowadays many patients with PHTP are discovered in their asymptomatic phase, especially in Western Europe and North Amerika(2) by screening for PTH and Ca2+ Very little is known about the molecular pathology/mechanisms associated with a chronic excessive action of PTH in different target tissues occurring during PHTP. The aim of the whole study was to describe changes in gene expression studied in bone marrow and skeletal muscle from patients with well defined and characterized, early PHPT before and after surgery when normalization of bone and biochemical markers had occurred. Each patient was used as their own control to describe molecular pathology expressed as mRNA profiles and suggest that the results may have clinical consequences reflecting chronic PTH receptor stimulation. My task was to quantify PTHR1 and PTHR2 in these patients using real time RT-PCR before and one year after surgery and at the same time to quantify the distribution of PTH-receptors also using real time RT-PCR in unrelated persons to define PTH-receptor target tissues.

Methods

Patients and biopsies

Seven arbitrarily chosen patients from 53.6 to 75.1 years of age (mean 60.3 years) without signs of organ affection, but with clinical biochemical evidence of mild PHPT were included.The diagnosis was established by elevated plasma PTH and ionized calcium, taking care to rule out accessory conditions known to affect the PTH/calcium balance. The patients were screened for interfering conditions such as cardiovascular and renal disorders, endocrine disorders and other bone pathology. Vitamin D-status (25(OH)D and 1,25(OH)2D) was evaluated before iclusion. The patients had normal serum creatinine levels. The patients underwent successful parathyroidectomy. Biopsies containing bone, bone marrow and skeletal muscle tissue were taken before and one year after surgery from opposite, but symmetrical places of os ileum to avoid woven bone from first biopsy, immediately frozen in liquid nitrogen and stored at –70C for RNA extraction. I quatified PTH receptors in these patients before and after surgery by using real time RT-PCR, this is a relative quantification compared to b-aktin. Distribution of PTHR1 and PTHR2 in (normal) muscle, heart and other tissues I studied on biopsies from other patients taken during surgery. The receptors in CNS were analysed in post mortem tissue from non-demented humans. The study was performed according to Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, and to the declaration of Helsinki II and approved by the Local Ethical Committee (Ethics ref no:19980012) Informed written consent was obtained from each PHPT participant and healthy persons before entry. Also examination of post mortem specimen fulfilled all formal requirements. Purification of heart-RNA and preparation of CDNA was done by me, with help from my colleges.

Purification of heart-RNA

I used heart-biopsies of children with di-George syndrom. RNA was purified with the aid of TRIZOL (Life Techologies, Gaithersburg, MD). Here is the procedure:

Reagents required ;

Chloroform

Isopropyl alcohol

75% Ethanol (in DEPC-treated water)

1.Homogenization of heart tissue:

Homogenize tissue samples in 1ml of TRIzol. The sample volume did/must not exceed 10% of the volume of TRIzol

2. Phase seperation

Incubate the homogenized samples for 5 min. at 30 C(degrees of Celsius), this is to permit the complete dissociation of nucleoprotein complexes. Add 0,2ml chloroform per 1 ml of TRIzol Reagent. Cap sample tubes securely. Shake tubes vigorously by hand for 15 sec. and incubate them at 30 C (degrees of Celsius) for 2-3min. Centrifuge the samples at no more than 12,000 x g for 15min. at 2-8 C (degrees of Celsius). Following centrifugation , the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase.

3. RNA precipitation

Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with isopropyl alcohol. Use 0,5ml of isopropyl alcohol per 1 ml of TRIzol. Incubate samples at 30 C (degrees of Celsius) for 10 min. and centrifuge at 12,000 x g for 10 min. at 2-8 C (degrees of Celsius). The RNA-precipitate, often invisible before centrifugation forms a gel-like pellet on the side and bottom of the tube.

4.RNA-wash

Remove the supernatant. Wash the RNA pellet once with 75% ethanol, adding at least 1 ml of 75% ethanol per 1 ml of TRIzol. Reagent used for the initial homogenisation. Mix the sample by vortexing and centrifuge at 7,500 x g for 5 min. at 2-8 C (degrees of Celsius)

5. Redissolving the RNA

At the end of the procedure, briefly dry the RNA pellet (air-dry for 5-10min). Dissolve RNA in RNase free water or 0,5% SDS solution (I used RNase free water) by passing the solution few times through a pipette tip, and incubating for 10 min. at 55-60 C (degrees of Celsius).

Preparation of CDNA

I used RNA from previous heart tissue to make CDNA by help from the enzyme reverse transkriptase? Equipment from Affymetrix was used. This was done in 2 major steps:

1.RNA/ T7-oligo(dT) Primer mix preparation

	Sample 1
Heart RNA	10 ul
T7-oligo(dT)primer 50uM	2ul
RNase free water	0ul
Total volume:	12ul

Mix the samples gently and vortex for 5 sec. Incubate at 70 C (degrees of Celsius) for 10min. Cool down the samples at 4 C (degrees of Celsius) for at least 2 min. Vortex again for 5 sec.

2. First-Strand Master Mix preparation. Here is the amount of the mix for one sample

	Master Mix
5x1st Strand Reaction Mix	4ul
DTT, 0,1M	2ul
DNTP, 10mM	1 ul
Total volum:	7ul

Vortex the Master Mix gently for 5 sec. 7ul of First Strand Master Mix is taken in each sample. Afterwards vortex the samples for 5 sec. Incubate at 42 C (degrees of Celsius) for 2 min. Add 1 ul Super Script II(reverse transkriptase) to each sample. Mix gently and vortex for 5 sec. Incubate the samples at 42 C (degrees of Celsius) for 1 hour. Cool down the samples at 4 C (degrees of Celsius) at least for 2 min. Centrifugate at for 5 sec just so that the fluid collets at the end of the tube.

In the same matter I did CDNA preparation from RNA from some samples of bonemarrow, muscle and different brain sections.

Real time RT-PCR

The distribution of PTHR1 and PTHR2 were analysed by real time RT-PCR employing the Light Cycler and the Fast Start Master SYBR Green kit (cat.no.2239264, Roche Diagnostics) according to manufacturers instructions. One sample from each organ/region from three different persons was analysed in triplicates. Cycling profile: 94 C (degrees of Celsius) for 5 min, then 40 cycles of (60 C for 30sec., 72 C for 30 sec., and 95 C fro 30 sec) and 3 min 72 C. Gene expression was normalized/compared to b-actin. The mRNA levels were tested in triplicates from 3 different persons The final results are presented in the figure 2 with bars illustrating mean+-SD

Gene expression was normalized to β-actin. Primers:

β-actin: Forw. GCTACAGCTTCACCACCACA Rev.

GCCATCTCTTGCTCGAAGTC

PTHR1: Forw. GTCCCTGAGACCTCGGTGTA Rev.

AGTACCGGAAGGTGCTCAAA

PTHR2: Forw. ATAGTGGGAGGCAGGGAGAT Rev.

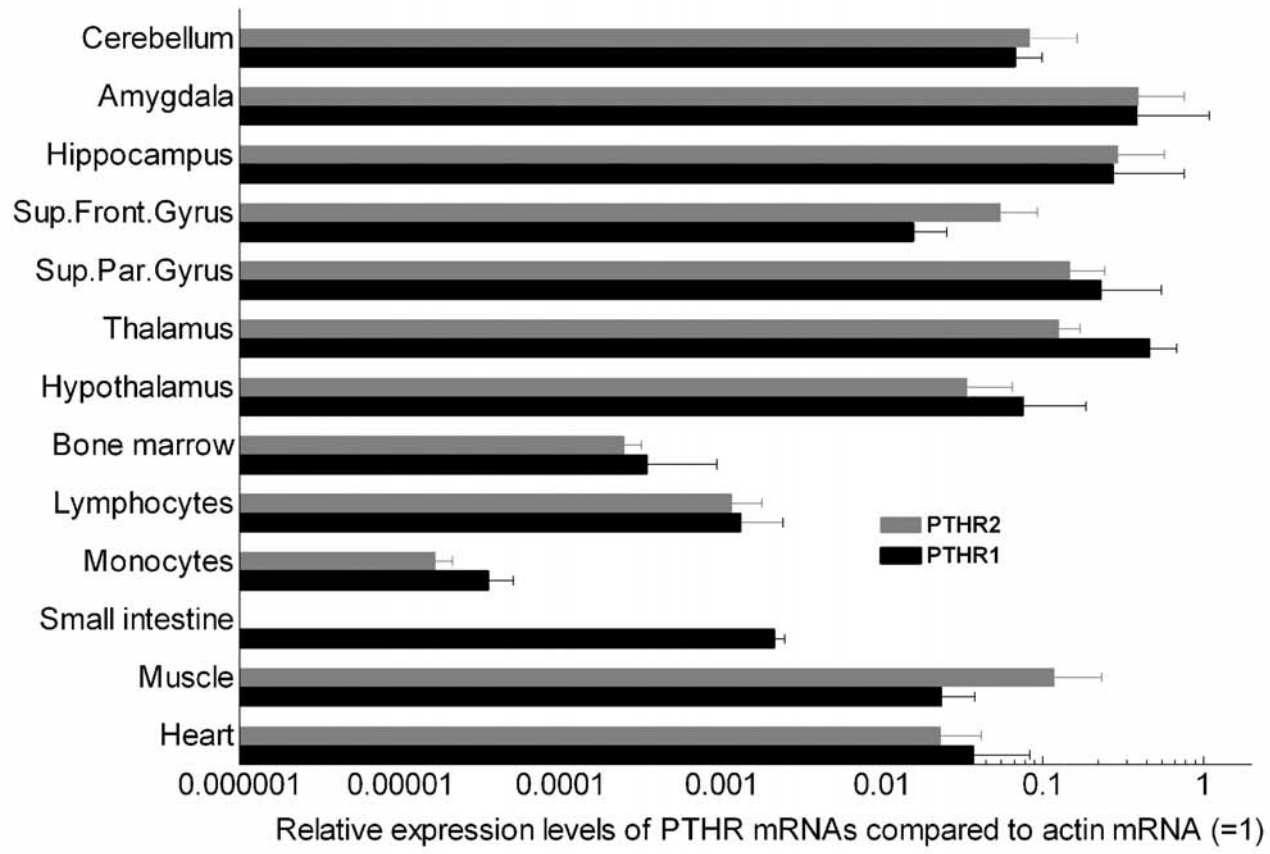
TTGGCATCCTTCAGTGTCTG

Results

TABLE 1 and FIGURE 2

Human PTH-receptor 1 and 2 expression is analysed by Real time PCR. 2µl of total RNA was analysed.
The data is given in ratio(N) to the expression level to b-actin that is set to be 1. The data is calculated out of crossing points(CT) of the different receptors.
ΔCT=patient – (b-aktin)
2∧(ΔCT)=R
1/R=N

TISSUE							
	CT (PTH-1)	CT (PTH-2)	CT (bAKTIN)	ΔCT (PTH1-b-AKTIN)	ΔCT (PTH2-b-AKTIN)	N(PTH1)	N(PTH2)
	X±SD	X±SD	X±SD	2^ΔCT	2^ΔCT		
Heart	28,45± 0,6	30,39± 0,56	22,5±0,05	5,96	7,89	0,016	0,0042
Muscle	33,56± 1,27	29,7± 0,31	28,1±0,05	5,46 44	1,6 3,03	0,023	0,33
Small Intestine	25,78± 0,04		17,12±0,05	8,66 404,5		0,0025	
Monocytes	31,68± 0,26	30,66± 0,94	14,55±0,03	17,3 161369	16,11 70728	0,000006	0,000014
Lymfocytes	30,73± 0,07	31,34± 1,18	14,85±0,06	15,88 60305	16,49 92042	0,000017	0,000011
Bone marrow	31,8± 0,12	28,45± 1,1	17,57±0,17	14,23 19215	10,88 1885	0,00005	0,00053
CNS							
Hypotalamus	25,07± 1,20	26,4± 1,25	22,39±0,43	2,68 6,4	4,01 16,11	0,156	0,0062
Thalamus	23,0± 0,57	25,26± 1,09	22,22±0,26	0,78 1,72	3,04 8,22	0,56	0,12
Sup.Par.Gyrus	22,54± 2,56	21,57± 0,58	19,97±0,19	2,57 5,94	1,6 3,03	0,17	0,33
Sup.Front. Gyrus	27,59± 0,58	24,96± 0,32	22,22±0,07	5,37 41,4	2,74 6,68	0,024	0,15
Hippocampus	21,20± 0,92	21,34± 0,58	20,70±0,13	0,55 1,46	0,64 1,56	0,68	0,64
Amygdala	21,95± 1,76	21,54± 0,03	21,64±0,57	0,31 1,24	-0,1 0,93	0,81	1,07
Cerebellum	24,77± 0,28	23,65± 0,58	21,43±0,34	3,34 10,13	2,22 4,66	0,099	0,215


Cerebellum
Amygdala
Hippocampus
Sup.Front.Gyrus
Sup.Par.Gyrus
Thalamus
Hypothalamus
Bone marrow
Lymphocytes
Monocytes
Small intestine
Muscle
Heart
PTHR2
PTHR1
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
Relative expression levels of PTHR mRNAs compared to actin mRNA (=1)

Fig, 2

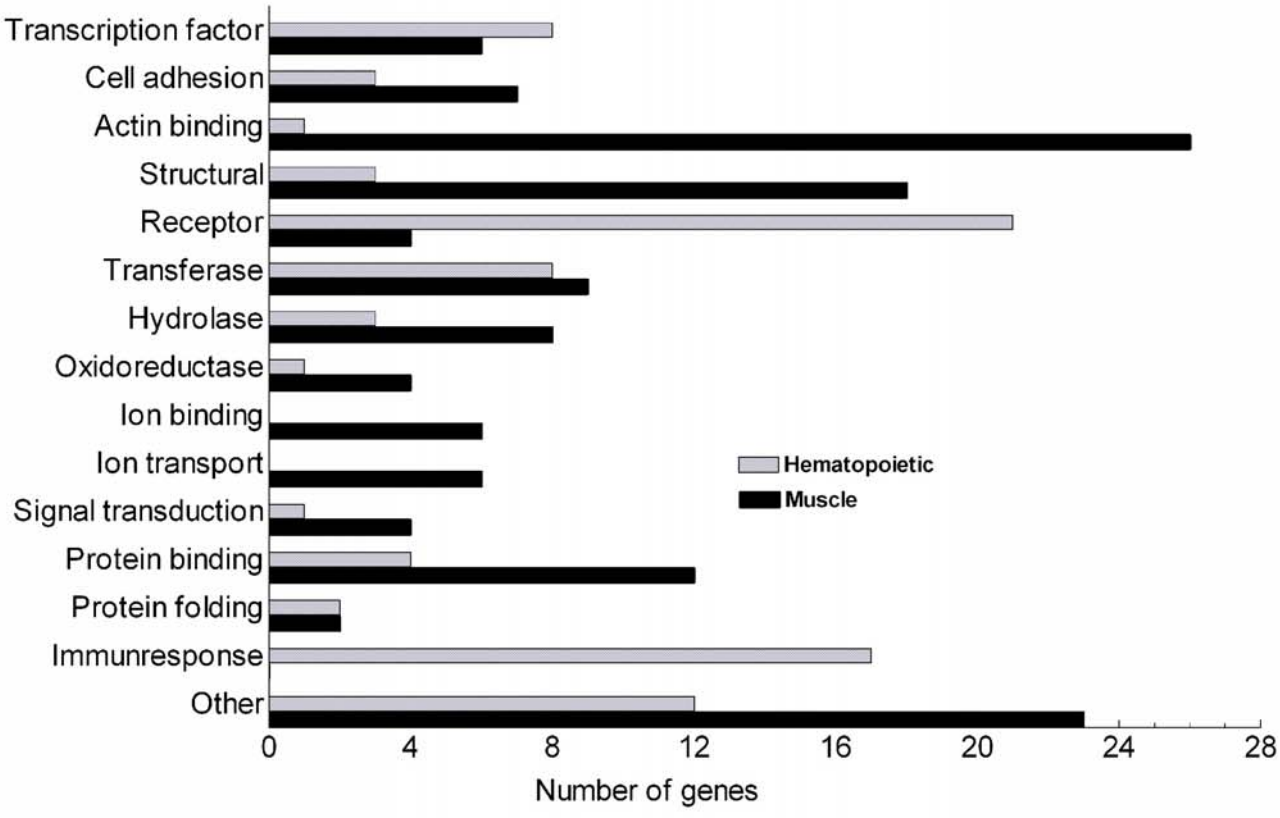

Transcription factor
Cell adhesion
Actin binding
Structural
Receptor
Transferase
Hydrolase
Oxidoreductase
Ion binding
Ion transport
Signal transduction
Protein binding
Protein folding
Immunresponse
Other
Hematopoietic
Muscle
0
4
8
12
16
20
24
28
Number of genes

Fig. 3

Quantification of PTH receptors in patients with PHPT

Before and one year after surgery

PATIENT:	CT PTH1(BEFORE)	CT PTH2(BEFORE)	CT B-AKT (BEFORE)	ΔCT(PTH1-B)	ΔCT(PTH2-B)
1	30,59± 0,018	32,93±0,404	22,07±0,03	8,52	10,86
2	28,34± 0,054	31,22±0,095	19,96±0,140	8,38	11,26
4	29,43±0,115	31,35±0,181	20,11±0,045	9,32	11,24
6	30,16±0,120	30,7±0,297	20,13±0,085	10,03	10,57

PATIENT:	CT PTH1(AFTER)	CT PTH2(AFTER)	CT B-AKT (AFTER)	ΔCT(PTH1-B)	ΔCT(PTH2-B)
1	30,55±0,0077	30,62±0,06	19,71±0,053	10,84	10,91
2	29,72±0,064	30,83±0,018	20,62±0,223	9,1	10,21
4	30,45±0,099	31,31±0,230	20,29±0,224	10,16	11,02
6	29,35±187	29,7±0,119	19,09±0,068	10,26	10,61

Discussion

Three types of PTH receptors (PTH/PTHrp receptor(PTHR1), the N-terminal PTH receptor 2 (PTHR2), and the C-PTH receptor(22,38,46,47)) have been isolated or partly characterized. Real time RT-PCR of PTHR1 mRNA showed a 2.0 ±0.9 fold increase in PHPT while PTHR2 mRNA expression was un-altered, confirming the Affymetrix data (35). These results show that. PTHR1 and PTHR2 mRNAs were both present in all human tissues and cells tested except for the small intestine where only PTHR1 mRNA was detected (Fig. 2). The abundant and uniform representation of the

two receptors mRNA in the CNS, especially Cerebellum, Amygdala, Hippocampus, Superior Frontal Gyrus, Superior Parietal Gyrus, Thalamus and Hypothalamus is noteworthy, reaching almost the level of actin (Fig. 2). This can be of great importance in explaining the different psychiatric manifestations in PHPT. Cardiac and skeletal muscle contains on average about 1/10 of the mRNA amounts present in the most enriched regions of the brain. All of the patients had high serum PTH while showing small or modest rises in serum Ca^{2+}. Since the median change in serum PTH and Ca^{2+} during disease was 5-fold and 1.25-fold respectively,it is tempting to regard PTH and not serum Ca^{2+}, as a major cause of the molecular de arrangement. In this way PTH action on the receptors in cardiac and skeletal muscle could be responsible for heart hypertrophy and muscular fatigue,myopathy and other symptoms rising from these structures.

The present study indicates that chronic PTH stimulation affects defined groups of mRNAs expressed in all target cells. It is therefore important to define the human target cells/tissues for PTH in order to understand the observed molecular changes in relation to clinical cell and organ pathology

Our study showed through Affymetrix profound changes observed in specific molecular patterns, and different molecular pathology For example the canonical pathways were affected, most significantly “Calcium Signaling”pathway in which 50 out of 172 mRNAs showed changed expression during disease This pathway is important in the contractile process of a muscle At the same time it probably involves also the cardiac β-adrenergic system present in cardiomyocytes. This could help explain the dys-regulation of cardiac function and development of left ventricular hypertrophy and failure representing the main cause of premature death in patients with PHPT (20, 36)

Expression of genes that regulate proliferation and differentiation of muscle related cells were altered in patients with PHPT

Affymeterix also showed us that different genes affecting the innate immune system were affected in patient with PHPT Expression of chemokine, complentent components and other genes were altered

This could be related to resistance to infections and increased prevalence and premature death of malignant diseases in PHPT (1, 3, 11, 13, 20, 32). A relationship between PHPT and infection is described being the 4th most prevalent cause of premature death (20). Also, high prevalence of H. pylori in patients with PHPT (11) and urinary tract infections in 26 % of 201 tested dogs with PHPT (13) point to an increased susceptibility to infections.

Our study suggests that there may be many different molecular mechanisms that may explain clinical consequences of PHPT,but that these are mediated by the excess PTH and its action on PTH receptors Patient with PHPT showed a great increase in PTHR1 mRNA, while the PTHR2 mRNA was unaltered during disease. The present results points to that early diagnosis and treatment of these patients may avoid irreversible molecular pathology. At the same time these results can give rise to further research.

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